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Survey For Necrosis Virus

In Aspen

FINAL REPORT OF RESEARCH PROJECT (INT-80-0014-CA)
CONDUCTED BY THE BROOKLYN BOTANIC GARDEN RESEARCH
CENTER IN COOPERATION WITH THE FOREST SERVICE,
INTERMOUNTAIN FOREST AND RANGE EXPERIMENT STATION

Project Title. - Survey for Necrosis Virus in Aspen by ELISA Techniques and Identification of New Viruses in Deteriorating Aspen Clones in the Rocky Mountains.

Objective. - In a previous study, aspen necrosis virus, a strain of tobacco necrosis virus, was identified in some deteriorating clones of aspen. The new serological technique of ELISA allows the relatively rapid and efficient detection of virus in trees without the need to isolate and purify the virus repeatedly. Antiserum has been made for the aspen necrosis virus, and preliminary testing of ELISA methods with aspen leaves was successful. Detection of the virus in aspen leaves by this method encouraged us to use ELISA in a survey for the presence of this and other viruses in both deteriorating and healthy aspen clones.

In the previous study, possibly one other virus was isolated from deteriorating aspen, but no identifications were made. These results, plus the recovery by Berbee of a flexuous rod virus from aspen in Wisconsin, suggested that additional attempts be made to isolate and identify new viruses in deteriorating aspen clones. If successful, then further surveys for the presence of viruses in aspen using the ELISA serological technique could be conducted.

The recovery of tobacco necrosis virus from aspen leaves was unusual because this virus is soil-borne, and it usually is restricted to plant roots. To determine if the aspen necrosis virus can be detected in soils from aspen sites, attempts were made to isolate the virus from samples of soil from deteriorating and healthy aspen clones.

Principal Investigators. - Dr. Craig R. Hibben, Research Plant

Pathologist, Brooklyn Botanic Garden Research Center, in cooperation with Dr. Robert F. Bozarth, Department of Life Sciences, Indiana State University.

Duration of Project. - September 15, 1980 to September 15, 1981. An extension (Amendment No. 1 to Supplement No. INT-80-14-CA) was granted from September 19, 1981 to March 31, 1982.

Procedure.

Aspen Collections. - Collections were made from five deteriorating and two healthy aspen clones in Logan Canyon, Utah, June 23 and 24, 1981. Leaf samples were harvested from five trees or three trees per clone. Five leaves per tree were cut in half along the midrib so that half of each tree sample could be tested by the ELISA method and the other half could be assayed for the presence of virus by mechanical transmission attempts. Additional leaves were harvested from each of the five trees per clone for more extensive mechanical transmission attempts. For all clones, the collections were biased towards the sampling of aspen showing foliar symptoms suggestive of virus infection (e.g. chlorotic spots, chlorosis or necrosis along veins, line patterns, stunting, etc.).

Root and soil samples were collected from three deteriorating and two healthy clones. Two trees per clone were sampled. For each tree, three root pieces (6-in. long by ½-to 1-in. diameter) and a composite (ca. 1 pint) soil sample dug from three locations around the tree were collected.

All leaf samples were held in the field in a portable styrofoam container cooled with ice. The leaf samples for ELISA tests were mailed (usually the same day collected) to Dr. Bozarth. The half-leaves plus some of the whole-leaves for mechanical transmission attempts were mailed to the Brooklyn Botanic Garden Research Center (BBGRC). The remaining leaves for mechanical

transmission attempts were held in a coldroom at the U.S.F.S. Station in Logan until transported back to the BBGRC in insulated mailers.

All root and soil samples were stored in the coldroom and later were mailed to the BBGRC.

Collections were made from four deteriorating and three healthy aspen clones along Salt River Pass (Rt. 89) in the Bridger National Forest, Wyoming; along Crow Creek Road (border of Wyoming and Idaho); and along Strawberry Canyon in the Caribou National Forest, Idaho, June 25 and 26, 1981. Root and soil samples were collected from two deteriorating and one healthy clone. The leaf, root, and soil samples were handled as described above.

Final leaf collections were made from four deteriorating and two healthy aspen clones along the Alpine Loop in the Uinta National Forest, Utah, and in the Strawberry Reservoir area (Rt. 40), June 28 and 29, 1981. The leaf samples were carried back to the BBGRC in insulated mailers, and half-leaves for the ELISA tests were mailed to Dr. Bozarth from there.

In addition to the above samples, leaves were similarly collected and processed from ten additional aspen which showed abnormal foliar or growth symptoms, as observed along roads or while surveying clones in the three collection sites.

A few aspen had the remnants of 1981 catkins. Catkins were harvested for mechanical transmission attempts.

Additional aspen leaf samples for ELISA tests were collected (Dr. Bozarth et al) in Indiana Dunes National Park, Indiana; Bendix Woods, Kalamazoo, Michigan; and Lake Geneva, Wisconsin, June 6-9, 1981. Ten clones, consisting of two clones at each of five sites, were sampled at each location. Clones were examined for foliar symptoms indicative of virus infection. Only

leaves with symptoms were collected for the tests. Harvested leaves were held in a cooled container in the field, and they were maintained at 4 C until processed.

ELISA.

Preparation of gamma-globulins for ELISA. - The μ-globulins were partially purified by precipitation with ammonium sulfate, equilibrated in half-strength PBS buffer, and washed through DEAE cellulose and buffer. Purified μ-globulin preparations with A_{280} of 1.4 were assumed to have a concentration of 1mg/ml. The purified μ-globulins were conjugated with alkaline phosphatase in the presence of .06% glutaraldehyde and stored at 4C with bovine serum albumin after thorough dialysis.

Serial dilution pre-tests were run on the antibodies, and it was found that for ELISA tests to be used in this survey, μ-globulin diluted 1:900 and conjugated globulin diluted 1:1200 gave optium results.

Aspen necrosis virus-globulin and tobacco ringspot virus-globulin were purified by the above procedure. Poplar mosaic virus antibody was provided by Dr. J. I. Cooper, University of Oxford, England. All three antibodies were conjugated by the above procedure.

Sources of purified virus. - Three viruses were used in this survey. Aspen necrosis virus (ANV) was propagated in cowpea primary leaves. Infected cowpea leaves (100-200g) were triturated in .01 M acetate buffer, pH 5, in a Waring blender. The extracts were filtered through cheesecloth, concentrated by differential centrifugation and centrifuged on sucrose density gradients in a Beckman Ti-15 zonal rotor for 2 hours at 39,000 rpm. The gradients were analyzed with an ISCO density-gradient fractionator and ISCO UA-5 monitor.

Tobacco ringspot virus (TOBRSV) was provided by Dr. C. R. Hibben.

Method. - Wells of a polystyrene microtiter plate were incubated with purified nonconjugated μ-globulin (1 ug/ml, 200 ul/well) for 2 hours at 37 C. One plate (plus one duplicate) was prepared for each of the three μ-globulins.

Leaf extracts were prepared by homogenizing leaf samples in a Waring blender with 15 mls of PBS-Tween-PVP buffer (.02 M phosphate, .15 M NaCl, .02% sodium azide, pH 7.4, containing .05% Tween-20 and 2% polyvinylpyrrolidone). The test samples (200 ul) were added to each well, incubated overnight at 6 C, and then washed three times with PBS-Tween-20. Conjugated μ-globulin (200 ul) was incubated in the plate wells for 3 hours at 37 C. The plates were washed three times. A 300 ul aliquot of substrate (p-nitrophenolphosphate) in substrate buffer (10% diethanolamine containing .02% sodium azide, pH 9.6) was incubated in each well. After one hour the positive nitrophenolate end-product was quantified by colorimetry (A_{405nm}). Those reactions giving obvious yellow color equal to or greater than 2X the average for the negative control samples in the same experiment were regarded as positive. All tests were duplicated, and the results were highly reproducible.

A control consisting of sample buffer substituted for the tests sample was used in every experiment. Also included on every plate was a positive control consisting of purified virus and a negative control consisting of an unrelated virus.

Mechanical transmission tests. - For each clone, both half-leaf and whole-leaf samples were tested for virus by several methods. The half-leaves were triturated in 0.01 M sodium diethyldithiocarbamate plus 0.02 M sodium thioglycollate in 0.05 M phosphate buffer, pH 8 (DIECA method). The extract was filtered through cheesecloth and centrifuged at 9g for 10 minutes. The supernate (and all preparations) was rubbed onto the following virus indicator plants previously dusted with carborundum: California blackeye cowpea (Vigna unguiculata), Marketer cucumber

(Cucumis sativus), Xanthi tobacco (Nicotiana tabacum), and Chenopodium amaranticolor. The supernate was then centrifuged at 30g for 3 hours, and a duplicate set of indicator plants was inoculated with the final high speed pellet resuspended in 0.05 M phosphate buffer, pH 7. Catkins were assayed by the DIECA method.

The following methods were used for the whole-leaf samples (leaves washed, air-dried, refrigerated at 4 C or frozen until assayed): DIECA method plus charcoal (5% wt/vol. added to low speed supernate, 3500g for 10 minutes, save supernate etc.), polyvinylpyrrolidone (PVP) method (leaves triturated in 2% PVP in 0.05 M phosphate buffer, pH 8, etc.), and the polyethylene-glycol (PEG) method (leaves triturated in 0.05 M phosphate buffer, pH 7, and equal volume of 20% PEG plus 0.3M NaCl in phosphate buffer added to low speed supernate, pellet from second low speed centrifugation resuspended in buffer and assayed on virus indicator plants). Most aspen clones, therefore, were tested for virus by four mechanical transmission methods.

The root pieces, after washing and storage at 3 C, were placed in plastic trays, covered with a mixture of moist peat-perlite-vermiculite (1:1:1), and maintained in the greenhouse to force root sprouts for additional mechanical transmission attempts.

The soil samples were tested for the presence of soil-borne ANV, an aspen isolate of tabacco necrosis virus, by the following method. Fifty ml. of 0.1 M phosphate buffer, pH 8, was added to 50g soil and placed on a shaker for 1 hour. The slurry was filtered through cheesecloth, centrifuged at 8g for 10 minutes, and the supernate, with 3 ml of 10% sucrose pipetted onto the bottom of each filled tube, was given centrifugation at 30 rpm for 3 hours. The final pellet was resuspended in buffer and assayed as described for the leaf extracts.

All inoculated virus indicator plants were maintained on greenhouse benches for 2 to 3 weeks for the development of symptoms. During the winter months, the indicator plants were held part of the time in a controlled environment growth chamber to encourgge more vigorous growth.

Results.

ELISA. - All leaf samples were triturated in 15 mls of sample buffer and 200 ul aliquots were pipetted into each well of the plate. Positive ELISA reactions were assessed by photometric measurement at 405nm. Color intensity was proportional to virus concentration. Wells ranged in color intensity from slightly positive (just over the negative criterion mark) to highly positive (over 2.00). So it is assumed that virus titer was greater in some samples than in others.

Out of thirty-three aspen samples collected from the Logan Canyon sites, twenty-nine showed a positive ELISA reading (above 0.100) for PMV, twenty-seven showed a positive ELISA reading (above 0.044) for TOBRSV, and no samples showed the presence of ANV (Table 1). All thirty-three samples showed the presence of at least one of the three viruses.

Out of fifteen samples collected at the Bridger National Forest and Crow Creek Road area (Table 2), eleven were ELISA positive (above .100) for PMV, eight were ELISA positive (above .044) for TOBRSV, and no samples were positive for ANV. One sample was negative for all three viruses.

The remaining western samples, i.e. Caribou National Forest, Unita National Forest, and Strawberry Reservoir area; all tested negative for all three viruses. These results are questionable, however, because the leaf material was badly deteriorated by the time the tests were conducted.

Out of ten samples from Indiana (Table 3), four showed a strong ELISA reaction (1.63-1.99) for PMV, four showed a weak ELISA reading (.36-.66) for ANV, and no samples showed a reaction for TOBRSV. The positive control for the samples made from purified virus diluted 1:300 showed positive reactions in the range of 2.06-2.43 . The negative control, which was an unrelated virus, showed a negative ELISA reading in the range of .003-.007 .

Out of ten samples collected from Wisconsin (Table 4), six

showed a positive ELISA reaction (1.43-1.94) to PMV, three showed a strong positive reaction (1.72-1.98) to ANV, and no samples showed a reaction to TOBRSV. The positive control for the samples showed positive readings in the range of 2.43-2.69. The negative control unrelated virus showed a negative ELISA reading in the range of .004-.009.

Out of ten samples collected in southern Michigan (Table 5), eight were slightly ELISA positive (.43-.98) for PMV, two were strongly positive (1.87-1.97) for ANV, and no samples were positive for TOBRSV. The positive controls in the samples ranged from 2.36-2.49, and the negative controls ranged from .003-.004. Eight of the ten samples tested positive for either PMV or ANV, and two samples were positive for both PMV and TOBRSV.

A comparison was made between ELISA results and the condition of the aspen clones in Logan Canyon, where deteriorating and healthy aspen clones have been well defined (Dr. Schier). The average absorbance (405nm) value for PMV was higher for deteriorating clones than for healthy clones, but when the values were analyzed for significance by the standard t-test, the differences were not significant at the 5% level. Two deteriorating clones (nos. 3 and 9), however, had significantly higher values for PMV than the two healthy clones. There were no significant differences in absorbance values for TOBRSV between the deteriorating and healthy clones.

It was difficult to correlate leaf symptoms with ELISA results in the western collections because of the predominance of leaf spotting by Marssonina populi, which hastened the deterioration of harvested leaves. The syndrome of chlorotic, feathery line patterns plus faint chlorotic spots and mottle was observed only in deteriorating clones 3 and 6 in Logan Canyon. (A similar observation was recorded in the 1975-76 study.) All the aspen sampled in Logan Canyon deteriorating clone no. 3 had the above

symptoms, and they also gave high absorbance values for PMV. Five symptomless trees in the two healthy clones, however, also gave similar high absorbance values for PMV. From these results the lack of foliar symptoms in aspen is not a reliable indicator for the absence of virus infection.

For the mid-western aspen (Indiana, Wisconsin, Michigan collections), there were some apparent correlations between foliar symptoms and positive ELISA tests. Eighteen out of 30 samples had trees with leaf symptoms consisting of mosaic, yellow-green mottling, veinal necrosis, and abnormalities in leaf size and shape. The same 18 samples gave positive ELISA tests for PMV. Nine out of 30 samples had trees with leaf symptoms consisting of yellow-green mottling, chlorosis along small veins, and chlorotic spots. These nine samples gave positive ELISA tests for ANV.

Mechanical transmission tests. - Most half-leaf samples (pressed between strips of paper towel and placed in plastic bags) arrived in good condition for virus recovery attempts. Many of the whole-leaf samples (placed in plastic bags with paper towel) that were mailed had turned partially to completely brown when received at the BBGRC. Especially discolored were young leaves and leaves infected with M. populi. Since the mailings took 2 to 4 days for delivery, it is likely that these samples deteriorated from the prolonged high temperature conditions. Most of the whole-leaf samples that were hand-carried (in insulated mailers) to the BBGRC remained in good condition. These leaf samples and the half-leaf samples were examined for symptoms, rinsed in water, air-dried, and refrigerated or frozen until assayed for virus.

The predominant leaf symptoms were chlorotic flecks and brown necrotic flecks and lesions, all probably infection stages of Marssonina populi and other leaf spot fungi. Very few aspen clones examined in 1981 did not show some evidence of infection

by leaf spot fungi. Other leaf symptoms occasionally observed were a chlorotic, feathery line pattern plus faint chlorotic spots and mottle (notably Logan Canyon deteriorating clones nos. 3 and 6), faint chlorotic spots, chlorosis along small veins, chlorotic mottle, and a dark brown necrosis along the main veins (notably Logan Canyon deteriorating clone no. 10).

Additional symptoms were randomly observed on individual aspen along roads and while surveying clones in the three collection sites, as follows: witches'-brooms along the trunk, entire tree uniformly chlorotic, chlorotic spots plus a high aphid population - along Crow Creek Road; foliage clumped towards the ends of twigs - many aspen along Strawberry Canyon and Alpine Loop; leaves with abundant fine chlorotic flecks, possibly insect injury - Logan Canyon; light green zones along the veins - Strawberry River area.

About 50 to 75 aspen of all sizes in the Timpooneke Campground off the Alpine Loop exhibited the unusual drooping symptoms described and studied by Livingston et al. (Plant Disease Reporter 63:923-927). Adjacent aspen clones showed no evidence of the drooping growth. It was interesting to note that chokecherry (Prunus virginiana) along the Alpine Loop road also appeared to have similar drooping branch symptoms. Chokecherry is an eastern host for a mycoplasma disease of peach.

The results of extensive mechanical transmission attempts with aspen leaf material did not corroborate the ELISA results. Of the three viruses tested by the ELISA method, only TOBRSV was recovered once from aspen leaves. This isolate came from a witches'-broom observed on a single tree along the Alpine Loop in the Uinta National Forest. It was identified by herbaceous host range, and by positive reactions against TOBRSV antiserum when partially-purified preparations (from cowpea trifoliates) were used in Ouchterlony agar double-diffusion tests.

Tobacco mosaic virus (TMV) appeared to have been recovered from Logan Canyon clones 2 and 4. The authenticity of these isolates is in doubt because we have experienced the occasional occurrence of TMV as a greenhouse contaminant. Although forest tree hosts have been reported for TMV, our results would have to be repeated before aspen could be considered a host of this virus.

We were unable to conduct mechanical transmission tests with aspen root sprouts. The root pieces were in some way injured during transit or while in cold storage, so that no root sprouts were produced in greenhouse tests.

Aspen necrosis virus was not recovered from the soil samples collected in Logan Canyon. This confirms earlier (unreported) results of no recovery of ANV directly from field soil. Trap crop experiments would perhaps be advisable in future work, whereby herbaceous test plants susceptible to ANV are grown in samples of field soil. The roots of the test plants would then be assayed for ANV by mechanical transmission methods.

Discussion. - The results demonstrate above all the sensitivity of the ELISA serological technique for detecting viruses in aspen. Although the results were not uniform between the mid-western and western clonal material, three viruses were detected in aspen in this study.

The ANV has been recovered from aspen in the west (Hibben et al., Forest Science 25:547-567) and in Wisconsin (Castello and Berbee, Phytopathology News 12:186). Our results extend the range of ANV in this host. It is surprising the ANV was not detected in Logan Canyon, where it was first reported in deteriorating clones. We propose to repeat ELISA tests for aspen in this area with fresh leaf samples collected in 1982.

The ELISA technique is used predominantly as an efficient means of surveying plant material for the presence of known viruses, rather than as a method for discovering new viruses in a host. From our results, PMV, a known pathogen of Populus species, was identified for the first time in mid-western and western aspen.

The TOBRSV also was unexpectedly detected in aspen for the first time. The PMV and TOBRSV identification, however, must be confirmed by additional ELISA or other serological tests, and, preferably, by at least occasional recovery of these viruses from trees showing positive ELISA tests. Supplementary attempts at isolating these viruses from selected aspen clones in the midwest and Logan Canyon locations are proposed for 1982.

The difficulty of recovering viruses from woody plants is recognized, and it is somewhat of a limiting factor in this research. From previous tree virus work, we feel confident that the series of mechanical transmission methods that we use are often successful in recovering viruses from woody plant material. It is possible that the generally negative results of the transmission attempts reported in this study, as well as the inconsistent results of the ELISA tests, were due to the maturity of the sampled leaves and/or the sometimes poor condition of the leaf samples after transit. Future sampling of aspen clones should concentrate on young leaves and better preservation of the harvested leaf samples.

Our results demonstrate that virus infection of aspen may be more prevalent than recognized before, but the possible role of viruses in aspen deterioration remains unclear. Although viruses have been detected in deteriorating aspen and in trees with foliar abnormalities, viruses were also identified in healthy appearing clones. No cause and effect relationship between virus infection and aspen deterioration has been demonstrated. Infectivity experiments with virus-free aspen material and purified virus preparations are necessary before the significance of these pathogens in aspen can be ascertained.

Table 1. ELISA Detection of Aspen Necrosis Virus (ANV), Poplar Mosaic Virus (PMV), and Tobacco Ringspot Virus (TOBRSV) in Deteriorating and Healthy Aspen Clones in Logan Canyon, Utah.

Samples		Absorbance at 405nm		
Clone[1]	Tree	ANV	PMV	TOBRSV
3	1	0	2.19	2.51
	2	0	2.62	2.43
	3	0	2.43	2.54
	4	0	2.38	.998
	5	0	2.48	.899
6	1	0	.094	1.78
	2	0	.100	1.43
	3	0	.108	2.34
8	1	0	2.35	1.11
	2	0	.068	1.01
	3	0	2.40	.987
	4	0	2.65	.213
	5	0	1.83	.012
9	1	0	2.83	.017
	2	0	2.14	.093
	3	0	2.42	.043
	4	0	2.41	.028
	5	0	2.41	.063
10	1	0	.412	2.00
	2	0	.548	2.62
	3	0	.081	2.53
B	1	0	.112	.031
	2	0	2.12	.238
	3	0	1.28	1.96
	4	0	2.42	.248
	5	0	2.34	.200
E	1	0	2.02	2.58
	2	0	.063	1.03
	3	0	2.15	1.62
	4	0	.998	1.81
	5	0	.134	1.81

[1] Dr. Schier's deteriorating (nos. 3, 6, 8, 9, 10) and healthy (B, E) clones.

Table 2. ELISA Detection of Aspen Necrosis Virus (ANV), Poplar Mosaic Virus (PMV), and Tobacco Ringspot Virus (TOBRSV) in Deteriorating and Healthy Aspen Clones in the Bridger National Forest and Along Crow Creek Road, Wyoming.

Samples		Absorbance at 405nm		
Clone[1]	Tree	ANV	PMV	AGV
1	1	0	2.40	.006
	2	0	1.70	.021
	3	0	2.45	.034
2	1	0	.684	1.06
	2	0	.328	.998
	3	0	2.00	1.10
3	1	0	.019	1.83
	2	0	.083	1.27
	3	0	.096	1.46
4	1	0	2.42	.031
5	1	0	.269	1.13
6	1	0	2.90	.084
A	1	0	.136	.013
	2	0	.100	.048
	3	0	2.40	.021

[1] Deteriorating (nos. 1, 2, 3) and healthy (A) clones. Clone 4 = tree with witches'-brooms, clone 5 = tree with heavy aphid infestation plus chlorotic spots, clone 6 = tree with chlorotic spots.

Table 3. ELISA Detection of Aspen Necrosis Virus (ANV), Poplar Mosaic Virus (PMV), and Tobacco Ringspot Virus (TOBRSV) in Aspen Clones in Indiana Dunes National Park, Indiana.

	Absorbance at 405nm		
Samples	ANV	PMV	TOBRSV
1	.003	.006	.004
2	.001	.006	.010
3	.982	.004	.006
4	.911	.001	.001
5	.006	.003	.010
6	.004	1.84	.005
7	.010	1.82	.003
8	.004	.008	.002
9	1.23	.011	.001
10	1.87	1.94	.006

Table 4. ELISA Detection of Aspen Necrosis Virus (ANV), Poplar Mosaic Virus (PMV), and Tobacco Ringspot Virus (TOBRSV) in Aspen Clones at Lake Geneva, Wisconsin.

	Absorbance at 405nm		
Tree	ANV	PMV	TOBRSV
1	.006	1.68	.002
2	.009	1.43	.008
3	.004	1.59	.003
4	.007	1.76	.003
5	.003	.007	0
6	.784	.005	.006
7	.989	.003	.002
8	.976	.004	.004
9	.006	1.78	.002
10	.002	1.94	.009

Table 5. ELISA Detection of Aspen Necrosis Virus (ANV), Poplar Mosaic Virus (PMV), and Tobacco Ringspot Virus (TOBRSV) in Aspen Clones in Bendix Woods, Kalamazoo, Michigan.

	Absorbance at 405nm		
Tree	ANV	PMV	TOBRSV
1	.007	.630	.005
2	.005	.549	.005
3	.005	.986	.005
4	.008	.945	.005
5	.003	.437	.005
6	.007	.520	.005
7	1.97	.007	.005
8	1.98	.003	.005
9	.007	.008	.005
10	.009	.003	.005

Figure 1. Microtiter Plate With Wells Showing Positive (darker) ELISA Reactions. Wells 2, 3 = PMV; 4, 5 = ANV; 6, 7 = TOBRSV. Well B = leaf samples 1, 2; C = 3, 4; D = 5, 6; E = 7, 8; F = 9, 10; G = control.